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SUPPRESSION OF INFLAMMATORY OEDEMA BY IBUPROFEN INVOLVING A MECHANISM INDEPENDENT OF CYCLO-OXYGENASE INHIBITION

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Abstract—The effects of a non-steroidal anti-inflammatory compound, ibuprofen, on experimentally-induced local oedema responses in rabbit skin were investigated. The accumulation of intravenously-injected ^{125}I-albumin was used to measure oedema. Two peptides were used to increase microvascular permeability: C5a des Arg, whose action is dependent on circulating polymorphonuclear leukocytes, and bradykinin which acts directly on vascular endothelial cells. The peptides were injected intradermally together with either arachidonic acid or PGE_2 to potentiate oedema formation. To study the cyclo-oxygenase inhibitory activity of ibuprofen, the effects of the mediators were potentiated by addition of arachidonic acid. To investigate whether the drug had effects independent of cyclo-oxygenase inhibition, PGE_2 was used to potentiate responses. Both local and intravenous ibuprofen suppressed oedema induced by bradykinin + arachidonic acid and C5a des Arg + arachidonic acid, which is consistent with suppression of cyclo-oxygenase in the skin. Local ibuprofen had no effect on responses to bradykinin + PGE_2 or C5a des Arg + PGE_2. Intravenous ibuprofen had no significant effect on responses to bradykinin + PGE_2 but, in contrast, had a marked inhibitory effect on responses to C5a des Arg + PGE_2. It is concluded that, in addition to its known cyclo-oxygenase inhibitory activity, ibuprofen can inhibit inflammatory oedema at clinically-relevant doses by an action on circulating polymorphonuclear leukocytes. These observations *in vivo* appear to be related to other observations on the effects of ibuprofen on polymorphonuclear leukocyte function *in vitro* and *ex vivo*. The observations described may also relate to the protective effects of ibuprofen on experimentally-induced myocardial infarction.

Local inflammatory oedema induced in the rabbit by intradermal injection of zymosan (boiled yeast cell walls) is thought to result from the extravascular generation of the complement derived polypeptide C5a acting synergistically with a vasodilator prostaglandin [1, 2]. C5a, as an intact molecule and in its biologically more stable form C5a des Arg, increases venular permeability and this is markedly potentiated by arteriolar dilators probably because of the resulting elevated hydrostatic pressure within venules [1, 3]. Non-steroidal anti-inflammatory compounds inhibit prostaglandin synthesis [4] and it has been proposed that an important anti-inflammatory action of these drugs is the suppression of oedema as a consequence of inhibition of vasodilator prostaglandins [5]. Accordingly, it has been shown that zymosan-induced oedema is suppressed by indomethacin; an effect wich is reversible using local vasodilator prostaglandins [1, 5, 6].

Unlike increased microvascular permeability induced by intradermal injections of histamine and bradykinin, responses induced by zymosan and C5a are abolished by depletion of circulating polymorphonuclear (PMN) leukocytes [7]. This has led to the hypothesis that extravascularly-generated C5a triggers a rapid (within 6 min) interaction between circulating PMN leukocytes and venular endothelial cells which, by some unknown mechanism, increases permeability to plasma proteins [7]. This concept has been supported by work from other laboratories [8–11].

If leukocyte-endothelial cell interactions are of general importance to inflammatory oedema, these observations provide a rational target for anti-inflammatory therapy. Recent reports of inhibition of PMN leukocyte responses (including aggregation, locomotion, enzyme release and the oxygen burst) by ibuprofen *in vitro* [12–19] and *ex vivo* [19] have led us to investigate if this drug may be able to suppress inflammatory oedema *in vivo* by a mechanism independent of inhibition prostaglandin synthesis.

In this paper we provide evidence from *in vivo* experiments that ibuprofen can suppress inflammatory oedema by two mechanisms: inhibition of the generation of endogenous vasodilator prostaglandins and inhibition of increased microvascular permeability. The latter effect was observed with increased permeability induced by C5a but not with that induced by bradykinin. This implies that ibuprofen can inhibit the interaction between the PMN leukocyte and the venular endothelial cell which results in increased permeability *in vivo*.

MATERIALS AND METHODS

Animals. Male specific pathogen-free New Zealand white rabbits (2–3 kg) were purchased from Froxfield (Hampshire, U.K.).

Materials. Prostaglandin E_2 (PGE_2), arachidonic acid and bradykinin triacetate were from Sigma

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Chemical Co. (Poole, Dorset, U.K.). Evans blue dye was from British Drug Houses (Poole, Dorset, U.K.). ^{125}I-human serum albumin (20 mg albumin per ml sterile isotonic saline, 50 μCi/ml) was from Amersham International (Amersham, Bucks, U.K.). Sagatal (pentobarbitone sodium, 60 mg/ml) was from May & Baker (Dagenham, Essex, U.K.). Steriflex (sterile, pyrogen-free isotonic saline solution) was from The Boots Co. PLC (Nottingham, U.K.)

We are grateful to Dr I. M. Hunneyball of The Boots Co. PLC, Nottingham, U.K., for the gift of ibuprofen dihydrate; Dr John Parry of Pfizer Central Research, Sandwich, Kent, U.K., for dazmegrel; and Merck Sharp & Dohme, Hoddesdon, Hertfordshire, U.K., for indomethacin.

Stock solutions of PGE_2 (1 mg/ml ethanol), bradykinin triacetate (1 mg/ml ethanol) and arachidonic acid (10 mg/ml ethanol) were stored at −25°. For arachidonic acid, an intermediate dilution (0.1 mg/ml) was made in 1.4% sodium bicarbonate solution. Working solutions were freshly prepared in saline on the day of the experiment. Ibuprofen solutions were freshly made up every day by dissolving 26.5 mg sodium ibuprofen dihydrate per ml of saline (equivalent to 20 mg ibuprofen per ml). Indomethacin (3 mg/ml) and dazmegrel (2 mg/ml) were dissolved in 1.4% sodium bicarbonate solution.

Preparation of C5a des Arg. Highly purified rabbit C5a des Arg was prepared from zymosan-activated serum by Dr P. J. Jose in our laboratory [1, 2]. The procedures used were: (1) cation exchange using CM-Sephadex C-25 at pH 5.9; (2) removal of high molecular weight protein by precipitation from 50% ethanol; (3) gel permeation using Sephadex G-100; (4) cation exchange HPLC using a TSK 535 CM column (7.5 × 150 mm) with a linear gradient of sodium chloride (100–600 mM) in 50 mM sodium phosphate buffer pH 6.3 and a flow rate of 0.5 ml/min.

C5a des Arg was stored at 1.36 mg/ml in 50 mM acetic acid at −25°. Intermediate dilutions (20×) in saline were neutralised with sodium hydroxide. Appropriate dilutions were made in saline.

Measurement of plasma protein leakage in the rabbit skin. Oedema formation in rabbit skin was measured over a 30 min period as the local accumulation of intravenously-injected ^{125}I-human serum albumin (5 μCi, mixed with 2 ml of 2.5% Evans blue dye in saline) as previously described [6]. Doses of C5a des Arg and bradykinin (mixed with arachidonic acid or PGE_2 where indicated) were injected intradermally in 0.1 ml volumes into the clipped dorsal skin of rabbits anaesthetised with pentobarbitone (30 mg/kg, i.v.). For local treatment, ibuprofen or indomethacin were injected intradermally, mixed with the oedema-inducing agents.

For systemic treatment animals were injected intravenously with saline (1 ml/kg), ibuprofen (20 mg/kg) or indomethacin (3 mg/kg) 15 min before intradermal injections. At the end of the experiment (i.e. 30 min after intradermal injections) a blood sample was taken by cardiac puncture into heparin (10 U/ml final concentration), animals were killed by an overdose of anaesthetic, the injected skin sites were punched out (17 mm diameter) and counted in a 12 head γ-counter (LKB Wallac Multigamma II 1260). Plasma albumin in each skin sample was expressed in terms of an equivalent volume of plasma by dividing the skin sample count by the count of 1 μl of plasma [6]. All intradermal injections were given according to a balanced site pattern and injection order was based on a Latin Square design.

Peripheral blood samples for total and differential white cell counts were taken from the marginal ear vein before, and at several time intervals after, intravenous injection of ibuprofen. May-Grunwald-Giemsa stain was used for differential counts.

In one internally-controlled experiment the effect of a thromboxane synthesis inhibitor, dazmegrel, was tested on oedema responses induced by mixtures of C5a des Arg + PGE_2. Test agents were given intradermally after intravenous radiolabelled albumin and the reaction allowed to proceed for 30 min. Dazmegrel (2 mg/kg) was then given intravenously and ten min later remaining skin sites were injected with the same combinations of test agents. Thirty min later rabbits were killed and leakage was quantitated as described above.

RESULTS

1. *Effect of local and systemic administration of ibuprofen on cyclo-oxygenase activity in the rabbit skin*

We have previously shown that indomethacin prevents the potentiation of bradykinin-induced protein exudation by arachidonic acid, by inhibition of the cyclo-oxygenase dependent formation of a vasodilator prostaglandin [5, 6]. In the present study we have employed the same technique to evaluate the cyclo-oxygenase inhibitory activity of ibuprofen *in vivo*. Ibuprofen (10^{-9}–10^{-7} moles/site) was injected intradermally together with mixtures of C5a des Arg (5×10^{-11} moles/site) and arachidonic acid (3×10^{-9} moles/site). Plasma exudation was measured over a 30 min period as the local accumulation of intravenously-injected ^{125}I-albumin, and the results are shown in Fig. 1. Little plasma exudation was apparent after intradermal injection of C5a des Arg or arachidonic acid alone when compared to control levels obtained with intradermal injections of saline. The mixture of C5a des Arg + arachidonic acid, however, produced a marked extravasation of plasma protein, and this was suppressed by local ibuprofen in a dose dependent manner. A 50% inhibition of the potentiation by arachidonic acid was obtained with 7×10^{-9} moles of ibuprofen (for comparison, 50% inhibition with indomethacin in this model was obtained with 5×10^{-10} moles).

PGE_2 (3×10^{-10} moles/site) potentiated the C5a des Arg induced oedema responses to the same extent as arachidonic acid (3×10^{-9} moles/site), but the potentiation by PGE_2 was not affected by local ibuprofen. PGE_2 alone induced little plasma exudation as shown. Almost identical responses were obtained when bradykinin (10^{-10} moles/site) was used as the permeability increasing agent instead of C5a des Arg (results not shown).

Figure 2 shows a comparison of the responses produced by intradermal injections of different doses of C5a des Arg or bradykinin mixed with a fixed dose

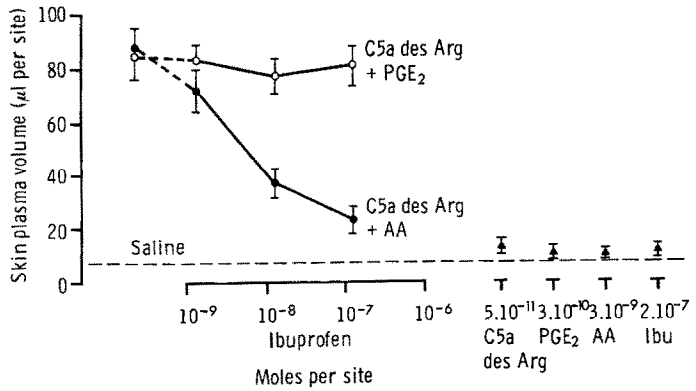

Fig. 1. Effect of local ibuprofen administration on cyclooxygenase activity in the rabbit skin. Plasma leakage was induced by intradermal injections of a fixed dose of C5a des Arg (5×10^{-11} moles/site) mixed with either arachidonic acid (3×10^{-9} moles/site) (●) or PGE_2 (3×10^{-10} moles/site) (○). Ibuprofen, at the doses shown, was mixed with the other agents before intradermal injections. The dashed line represents the control level determined by intradermal injection of saline. Closed symbols (▲) show the leakage response to intradermal injections of C5a des Arg, arachidonic acid (AA), PGE_2 or ibuprofen (ibu) alone. For experimental details, see Materials and Methods. Each point represents the mean ± S.E.M. of 6 replicate injections.

of arachidonic acid (3×10^{-9} moles/site) in animals treated systemically with ibuprofen (20 mg/kg intravenously, 15 min before intradermal injections) or control animals given an equal volume of saline. At this dose ibuprofen inhibited responses to C5a des Arg + arachidonic acid and bradykinin + arachidonic acid. Potentiation was inhibited by 72% and 58% respectively. C5a des Arg (5×10^{-11} moles/site) bradykinin (10^{-10} moles/site) or arachidonic acid (3×10^{-9} moles/site) alone produced negligible exudation in comparison with mixtures of C5a des Arg + arachidonic acid or bradykinin + arachidonic acid.

Similar results were obtained using intravenous indomethacin at 3 mg/kg in place of ibuprofen. Responses to C5a des Arg + arachidonic acid and bradykinin + arachidonic acid were 90 ± 9 and 102 ± 12 μl/site in control animals compared to 33 ± 5 and 39 ± 7 μl in animals treated with indomethacin (N = 5 rabbits, agonists at the same doses as above).

2. *Suppression of oedema formation by ibuprofen, independently of cyclo-oxygenase inhibition*

As in the experiments described in the previous section, ibuprofen (20 mg/kg) was injected intravenously 15 min before local oedema responses were initiated by intradermal injections of C5a des Arg or bradykinin. In these experiments however, plasma leakage induced by C5a des Arg and bradykinin

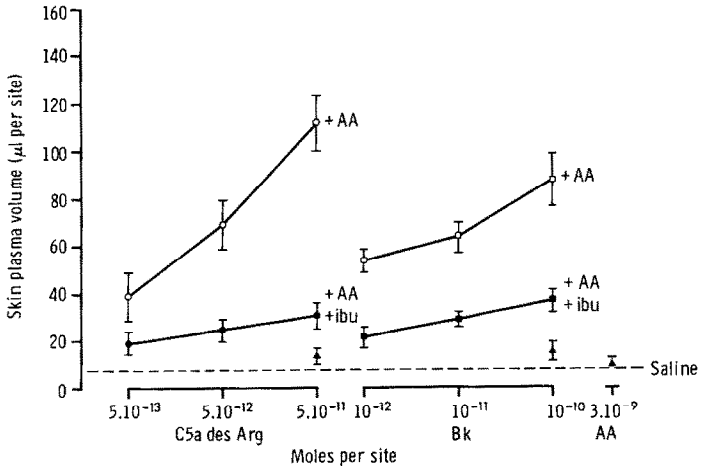

Fig. 2. Oedema formation in rabbit skin induced by mixtures of C5a des Arg + arachidonic acid (AA) and bradykinin (Bk) + arachidonic acid (AA) to show the cyclo-oxygenase inhibitory activity of intravenous ibuprofen (ibu, 20 mg/kg). The symbols represent: oedema responses to mixtures in animals given intravenous saline (1 ml/kg) (○□); responses to intradermal injection of C5a des Arg, bradykinin or arachidonic acid (AA) alone in animals given intravenous saline (▲); and responses in animals treated with ibuprofen (20 mg/kg) (●■). The dashed line represents the control level determined by intradermal injection of saline. For experimental details, see Materials and Methods. Results are the mean ± S.E.M. for 6 rabbits.

was potentiated by simultaneous injection of the vasodilator PGE_2, instead of its precursor arachidonic acid. As shown in Fig. 3, ibuprofen did not suppress oedema responses evoked by mixtures of bradykinin + PGE_2. A slightly higher response was generally seen in ibuprofen treated animals, although this did not reach the level of statistical significance. In contrast, plasma leakage induced by C5a des Arg + PGE_2 was markedly suppressed by systemic ibuprofen and this was statistically significant at the two higher doses of C5a des Arg tested (5×10^{-11}, 5×10^{-12} moles/site) ($P < 0.05$ Wilcoxon rank sum test). The top doses of C5a des Arg or bradykinin alone, as well as PGE_2 alone, induced only little plasma exudation.

In animals treated with intravenous indomethacin (3 mg/kg) responses to mixtures of C5a des Arg (5×10^{-11} moles/site) + PGE_2 (3×10^{-10} moles/site) were not different from responses in control animals given intravenous saline (1 ml/kg), i.e. 129 ± 25 μl/site compared with 123 ± 23 μl/site (N = 5 rabbits).

Similarly, intravenous injection of the thromboxane synthesis inhibitor dazmegrel (2 mg/kg) did not reduce leakage responses to C5a des Arg (5×10^{-11}, 5×10^{-12}, 5×10^{-13} moles/site) + PGE_2 (3×10^{-10} moles/site), i.e. 180 ± 20, 90 ± 12, 48 ± 7 μl/site compared with 164 ± 20, 105 ± 8 and 57 ± 6 μl/site after intravenous injection of dazmegrel (N = 6 sites). This dose of dazmegrel has been shown to be an efficient inhibitor of thromboxane synthesis in the rabbit [20].

Total and differential white cell counts in peripheral blood revealed no significant changes in the number of circulating neutrophils after intravenous injection of ibuprofen (20 mg/kg). Total white cell counts in these animals before, 15 and 45 min after ibuprofen were respectively 7.5 ± 1.5, 7.6 ± 1.6 and $7.6 \pm 1.3 \times 10^6$ cells/ml. PMN leukocyte counts at these time points were respectively 2.9 ± 0.7, 2.8 ± 0.6 and $2.8 \pm 0.7 \times 10^6$ cells/ml.

DISCUSSION

The results show that ibuprofen, administered locally or systemically, suppresses oedema induced by intradermal injections of the microvascular permeability-increasing peptides, C5a des Arg and bradykinin, when their effects are potentiated by arachidonic acid. This demonstrates how the known cyclo-oxygenase-inhibitory activity of ibuprofen can suppress inflammatory oedema by inhibiting the conversion of liberated endogenous arachidonic acid to vasodilator, oedema-potentiating prostaglandins, PGE_2 or PGI_2. This is consistent with earlier observations using indomethacin [5, 6]. Local ibuprofen had no effect on oedema induced by C5a des Arg or bradykinin when in combination with exogenous PGE_2, which demonstrates that the extravascular generation of cyclo-oxygenase products (e.g. thromboxane A_2) is not involved in the permeability responses to the peptides.

In contrast to the effects of local ibuprofen, intravenously-injected ibuprofen, at a dose within the clinical range, suppressed local oedema induced by C5a des Arg + PGE_2, without affecting responses to bradykinin + PGE_2. As the former response is dependent on circulating PMN leukocytes, whereas the latter is PMN-independent [7], these results suggest that systemic ibuprofen can inhibit the interaction between the leukocyte and the venular endothelial cell which is a prerequisite of increased permeability induced by C5a des Arg. This is supported by other observations that systemic ibuprofen inhibits increased microvascular permeability induced by the chemoattractants formyl-methionyl-

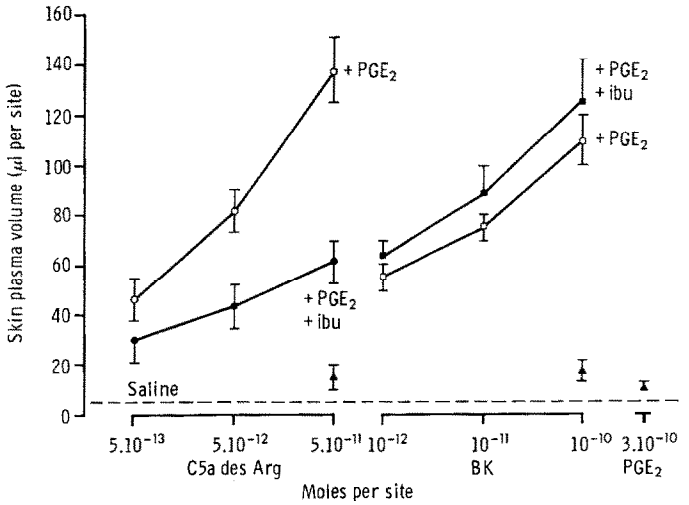

Fig. 3. Oedema formation in rabbit skin induced by mixtures of C5a des Arg + PGE_2 and bradykinin + PGE_2 to show the cyclo-oxygenase independent effect of intravenous ibuprofen (ibu, 20 mg/kg). The symbols represent: oedema responses to mixtures in animals given intravenous saline (1 ml/kg) (○□); responses to intradermal injection of C5a des Arg, bradykinin or PGE_2 alone in animals given intravenous saline (▲); and responses to mixtures in animals treated with ibuprofen (20 mg/kg) (●■); and the dashed line represents the control level determined by intradermal injection of saline. For experimental details, see Materials and Methods. Results are the mean ± S.E.M. for 6 rabbits.

leucyl-phenylalanine (Hellewell, Yarwood & Williams: unpublished observations) and leukotriene B_4, but not the response induced by histamine (Rampart and Williams: unpublished observations).

The recent observations of inhibitory effects of ibuprofen on PMN leukocytes *in vitro* [12–19] and *ex vivo* [19] suggest that the primary effect of the drug in inhibiting microvascular permeability *in vivo* may also be on the PMN leukocyte. Indeed, it has been shown that ibuprofen can inhibit ^{51}Cr release from cultured endothelial cells in contact with activated PMN leukocytes *in vitro* [18]. Whether this is strictly analogous to PMN leukocyte/endothelial cell interactions triggered by local extravascular chemoattractants *in vivo*, as in our experiments, remains to be determined.

It is of interest that systemic ibuprofen inhibited oedema induced by C5a des Arg + PGE_2, whereas local ibuprofen was ineffective. Locally-injected ibuprofen would be expected to diffuse into the microvessel lumen, therefore the concentration attained locally in the blood may be insufficient to affect PMN leukocytes in transit through the tissue. Alternatively, the leukocytes may require a time of pre-exposure to the drug before inhibition is apparent. No systemic effects of the drug would be expected using local ibuprofen as the total amount injected intradermally in the animal was <1% of the intravenous dose.

An involvement of a cyclo-oxygenase metabolite of arachidonic acid in increased microvascular permeability induced by the PMN leukocyte cannot be entirely ruled out. This would be the simplest explanation for the effect of systemic ibuprofen. However, several pieces of evidence militate against this possibility. Firstly, in these and previous experiments [5, 6] we have tested arachidonic acid, PGG_2, PGE_2, PGI_2, $PGF_{2\alpha}$, PGD_2 and 6-oxo-$PGF_{1\alpha}$ and found each of them to be inactive alone in increasing microvascular permeability, except at very high doses. Thromboxane A_2 remains a possible endogenous mediator in these experiments (thromboxane B_2 is inactive: Williams, unpublished observations). However, this is unlikely for two reasons. Firstly, as shown here, intravenous indomethacin, at a dose with comparable effects on cyclo-oxygenase, failed to inhibit oedema responses to C5a des Arg + PGE_2. Secondly, an intravenously-injected thromboxane synthesis inhibitor, dazmegrel, was also ineffective.

Some non-steroidal anti-inflammatory compounds have been shown to inhibit receptor binding of FMLP on PMN leukocytes *in vitro* [21–23]. Ibuprofen may be acting in this way in our *in vivo* experiments, although it is unlikely that the drug would equally inhibit responses to C5a des Arg, FMLP and leukotriene B_4 by this mechanism.

Some experiments have implicated PMN leukocyte-derived reactive oxygen species in microvascular lung injury induced by intravenously-injected leukocyte activators [24–27]. Recently, we have found evidence that the microvascular response to a local extravascular chemoattractant may also involve activated oxygen species, in that intravenous catalase inhibits local oedema induced by such chemoattractants when injected intradermally [28]. If such a mechanism is involved in the response to intradermal C5a des Arg, it could be that ibuprofen inhibits the generation or action of products of the respiratory burst *in vivo*.

It seems likely that our observations are related to those showing that ibuprofen suppresses experimentally-induced myocardial infarction, a reaction which involves the accumulation of PMN leukocytes [18, 29, 30].

It is to be hoped that, in the future, it will be possible to relate more closely the mechanisms involved in microvascular responses to chemically-defined agents, as in our experiments, and the more complex events occurring during tissue injury, as in myocardial infarction.

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